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Polydnavirus integration in lepidopteran host cells in vitro

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Abstract

The long-term persistence of polydnavirus (PDV) DNA in infected lepidopteran cell cultures has suggested that at least some of the virus sequences become integrated permanently into the cell genome. In the current study, we provide supportive evidence of this event. Cloned libraries were prepared from two different *Lymantria dispar* (gypsy moth) cell lines that had been maintained in continuous culture for more than five years after infection with *Glyptapanteles indiensis* PDV (GiPDV). Junction clones containing both insect chromosomal and polydnaviral sequences were isolated. Precise integration junction sites were identified by sequence comparison of linear (integrated) and circular forms of the GiPDV genome segment F, from which viral sequences originated. Host chromosomal sequences at the site of integration varied between the two *L. dispar* cell lines but virus sequence junctions were identical and contained a 4-base pair CATG palindromic repeat. The GiPDV segment F does not encode any self-replication or self-insertion proteins, suggesting a host-derived mechanism is responsible for its in vitro integration. The chromosomal site of one junction clone contained sequences indicative of a new *L. dispar* retrotransposon, including a putative reverse transcriptase and integrase located upstream of the site of viral integration. A potential mechanism is proposed for the integration of PDV DNA in vitro. It remains to be seen if integration of the virus also occurs in the lepidopteran host in vivo.

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1. Introduction

Polydnaviruses (PDVs), unique mutualistic insect viruses found in some parasitic Hymenoptera, are characterized by their unusual multi-segmented closed circular dsDNA genomes. PDVs are considered as non-traditional viruses because their replication has been detected only in oviduct calyx cells within the female parasitoid reproductive tract. PDVs are injected along with eggs by the parasitic wasps into their lepidopteran host hemocoel during oviposition. Inside the larval host, PDVs infect cells but do not replicate. Their genomes are evidently transcriptionally active (Blissard et al., 1986; Strand et al., 1992) within the host, and their transiently expressed gene products function to suppress the normal immune activity and regulate development and physiology of the host (Asgari et al., 1997; Blissard et al.,

1986; Lavine and Beckage, 1995; Lawrence and Lanzrein, 1993; Stoltz, 1993; Summers and Dibb-Hajj, 1995; Strand and Pech, 1995), presumably under the control of larval host factors that are not well understood. No response is mounted to eliminate the parasitoid eggs and they are allowed to develop, hence PDVs play essential roles in the parasitoid life cycle (Stoltz, 1993).

The two recognized PDV genera, ichnoviruses (IVs) and bracoviruses (BVs) associated with parasitoid wasps of Ichneumonidae and Braconidae, respectively, differ in their particle morphology, physiology, molecular characteristics, and host range (Stoltz and Whitfield, 1992; Stoltz et al., 1995; reviewed in Webb, 1998). Genetic studies have indicated that both IVs and BVs share the property of vertical transmission through the germ line (Stoltz, 1990; Stoltz et al., 1986), through integration into their respective wasp host genomes as proviruses (Fleming and Summers, 1990; Fleming and Summers, 1991; Fleming, 1991; Gruber et al., 1996; Savary et al., 1997; Stoltz, 1990; Stoltz, 1993; Stoltz et al., 1986; Wyder et al., 2002). Evidence for transmission through proviral genome integration has been shown for IVs

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associated with *Campoletis sonorensis* (the IV type species) (Fleming and Summers, 1986; Fleming and Summers, 1990) and *Hyposoter fugitivus* (Xu and Stoltz, 1991) and for BVs associated with *Chelonus inanitus* (Gruber et al., 1996; Wyder et al., 2002) and *Cotesia congregata* (Savary et al., 1997). The viral sites of proviral integration are marked by terminal repeats, which vary in length and homology with each PDV studied, and markedly between IVs and BVs (Fleming and Summers, 1991; Cui and Webb, 1997; Gruber et al., 1996; Wyder et al., 2002). PDV circular genome segments appear to be produced by excision at these repeats from the parasitoid genome, by a mechanism similar to those used by transposable elements, retroviruses, and retrotransposons (reviewed in Webb, 1998).

Studies concerning the fate of PDVs within their natural lepidopteran hosts have primarily focused on virus-specific gene expression and the physiological changes associated with parasitism (Stoltz, 1993) and have been conducted in vivo. A few studies have examined the fate of PDVs and the effects of PDV infection in vitro. To date, three PDVs have been described and shown to persist over time, maintained in vitro in infected cell populations derived from lepidopterans. Two are the IVs associated with *Hyposoter fugitivus* (HfPV) and *Hyposoter didymator* (HdIV). HfPV was used to infect a cell line derived from *Lymantria dispar* (Ld652Y), a non-permissive host of the parasitoid (Kim et al., 1996). HdIV was used to infect several cell lines derived from host *Spodoptera littoralis* haemocytes, as well as non-preferred hosts of the parasitoid *Spodoptera frugiperda* (SF9) and *Trichoplusia ni* (High Five) (Volkoff et al., 1999, 2001). The third is the BV associated with *Glyptapanteles indiensis* (GiPDV) in several cell lines derived from host *L. dispar* (McKelvey et al., 1996; Gundersen-Rindal and Dougherty, 2000) and non-preferred hosts of the parasitoid, including several lepidopteran (*T. ni*, *S. frugiperda*, *Plodia interpunctella*, and *Heliothis virescens*) and a coleopteran (*Diabrotica undecimpunctata*) cell lines (Gundersen-Rindal et al., 1999). In the examples of persistent HfPV and GiPDV infection, cytopathic effects were observed in cells initially upon infection and for several weeks, followed by a period of cellular recovery in which the cell population morphology and growth patterns returned essentially to normal. In all three examples, viral sequences were maintained in the recovered and apparently transformed cell lines in association with high molecular weight cellular DNA of presumed chromosomal origin. Furthermore, the viral sequences were readily detectable after 18 up to 36 months, even after 60 months for HdIV/SF9 and GiPDV/LdEp, of routine cell subculture. For HdIV, an uncharacterized K-gene was shown to be transcribed and expressed in long-term transformed *Spodoptera* and *Trichoplusia* cells, while other HdIV genes were not transcribed (Volkoff et al., 2001). Likewise,

several GiPDV-encoded genes in long-term transformed LdEp cells appear to be transcribed (Gundersen-Rindal, unpublished results).

With the exception of one site from GiPDV/LdEp (Gundersen-Rindal and Dougherty, 2000), neither PDV in vitro integration border sites containing virus and chromosomal sequence have been isolated nor potential means or mechanisms for integration examined, for any PDV systems. Because our laboratory is interested in virus-mediated in vitro cell transformation, chromosomal and viral junction sequences of a GiPDV circular genome segment integrated in vitro in two different *L. dispar* cell lines were isolated and analyzed to investigate the nature of PDV in vitro integration.

2. Materials and methods

2.1. GiPDV-transformed *L. dispar* cell lines

L. dispar cell lines originally derived from embryonic tissue (IPLB-LdEp) and pupal ovaries (IPLB-Ld652Y) were infected with GiPDV in vitro as previously described (McKelvey et al., 1996). Total genomic nucleic acids were extracted from non-infected and GiPDV-infected IPLB-LdEp (IPLB-LdEp/Gi) and IPLB-Ld652Y (IPLB-Ld652Y/Gi) cells, after greater than 250 passages, by standard techniques (Ausubel et al., 1994).

2.2. Isolation of GiPDV DNA and GiPDV genomic segment clones

Female braconid *G. indiensis* parasitic wasps were dissected and calyx fluid containing PDVs extracted for nucleic acid as previously described (Beckage et al., 1994). A GiPDV plasmid clone library was generated using the method described by Albrecht et al. (1994) to isolate complete GiPDV circular genomic segments. The library was screened to identify GiPDV circular segments, including p157 analyzed in this study, capable of persisting in infected *L. dispar* cell in vitro as previously described (Gundersen-Rindal and Dougherty, 2000). The full ds circular segment represented by clone p157 was recently designated as segment F of the GiPDV genome (Chen and Gundersen-Rindal, in press) and is referred to as segment F/p157 throughout.

2.3. Restriction analysis of GiPDV-transformed *L. dispar* cell DNAs for GiPDV sequences persisting in vitro

Digoxigenin-labeled plasmid DNA from GiPDV segment F/p157 was used as a hybridization probe to analyze transformed IPLB-LdEp/Gi cell DNA for evidence of integrated GiPDV viral sequences. For Southern blot, cloned segment F/p157 (1 μ g) and IPLB-LdEp/Gi (5 μ g

each) DNAs were digested separately with restriction endonucleases *Bam*HI, *Kpn*I, *Pst*I, *Sst*I, and *Xba*I (Invitrogen, Carlsbad, CA), electrophoresed through a 0.8% agarose gel, transferred to nylon membrane (Southern, 1975), and probed. Uncut GiPDV (0.5 ug) and IPLB-LdEp (5 ug) genomic DNAs were included as positive and negative controls, respectively. The temperature for hybridization was 48 °C. Annealed DNAs were visualized using the chemiluminescent detection reagent disodium 2-chloro-5-(4 methoxyspiro [1,2-dioxetane-3,2'-(5'-chloro)-tricyclo [3,3,1,13,7]decan]-4y1)phenyl phosphate(CSPD) (Roche Biochemicals, Indianapolis, IN). GiPDV segment F/p157 was sequenced by a combination of primer walking and using the GPS-1 genome priming system (New England Biolabs, Beverly, MA) on an ABI310 automatic sequencer (Applied Biosystems, Foster City, CA). A restriction map based on restriction endonuclease recognition sites was generated from the sequence using the MapDraw component of DNASTAR software package (Madison, WI). The putative site of viral integration for this segment was located on the map.

2.4. Isolation and analysis of chromosomal:viral integration junctions

Two genomic libraries were derived from GiPDV-infected Ld cells. The IPLB-LdEp/Gi clone p7-54, containing an integration junction, was derived by subcloning from an IPLB-LdEp/Gi lambda phage library as described previously (Gundersen-Rindal and Dougherty, 2000). The IPLB-Ld652Y/Gi library was derived by ligating *Bam*HI partially-digested DNA, filled in using the Klenow fragment of DNA polymerase I, into the *Xho*I site of vector Lambda Fix II (Stratagene, Cedar Creek, TX). Clones hybridizing to digoxigenin-labeled GiPDV were isolated by plaque lift. Clones were analyzed by restriction digestion followed by Southern hybridization using digoxigenin-labeled total GiPDV and IPLB-Ld652Y DNAs separately as probes. Lambda clones containing fragments that hybridized with both viral and cellular probes were sub-cloned by inserting either *Hind*III or *Xba*I fragments into the pGEM3zf(+) plasmid vector (Promega Corp., Madison, WI). Sub-clones were screened by reciprocal hybridization using labeled GiPDV and IPLB-Ld652Y DNAs as probes. Several sub-clones containing both viral and chromosomal sequences were isolated and analyzed. Clone Ld16XE11 from this library, as well as p7-54 from the IPLB-LdEp/Gi library, were sequenced after using the GPS-1 genome priming system (New England Biolabs) on an ABI310 automatic sequencer (Applied Biosystems) and determined to originate from the same PDV genome segment. Sequences were assembled using the SeqManII component of the DNASTAR software package (Madison, WI). The GiPDV sequences

(integrated) of IPLB-LdEp/Gi clone p7-54 and IPLB-Ld652Y/Gi clone L16XE11 were compared with GiPDV sequences (circular) obtained from GiPDV circular DNA segment F/p157 and integration junctions identified. All sequences were analyzed for homologies to known sequences in GenBank using BLAST (Altschul et al., 1990, 1997). Sequences for junction clones p7-54 (updated sequence) and L16XE11 have been deposited in GenBank under accession numbers AF198385 and AY162267, respectively.

3. Results

3.1. GiPDV-transformed *L. dispar* cell lines

Infection of two *L. dispar* cell lines derived from embryonic tissue with GiPDV in vitro was previously described (McKelvey et al., 1996). The GiPDV-infected IPLB-LdEp (IPLB-LdEP/Gi) and Ld652Y (Ld652Y/Gi) cells utilized in these experiments had been routinely passed in the laboratory at least 250 times when genomic DNA was extracted for libraries, indicating that the GiPDV viral sequences were stably maintained as a permanent part of the replicating cells and the cells were, in fact, transformed.

3.2. GiPDV DNA segment maintained in infected transformed Ld cells in vitro

Clone p157 was 18.6 kb in size as determined by restriction and full sequence analysis and represented a complete dsDNA circular segment of the GiPDV genome as previously described. In characterization of the GiPDV genome, the viral segment represented by clone p157 was designated as GiPDV genome segment F (Chen and Gundersen-Rindal, submitted). Segment F is presented graphically (Fig. 1A) in the orientation in which it was originally cloned and sequenced. The same restriction profile is generated by analysis of the reverse complement or opposite orientation of the circle, and the full circular segment F has been cloned in both orientations (data not shown).

Each PDV-infected insect cell line examined to date for in vitro persistence of polydnviral sequences has indicated that part, but not all, of the viral genome is maintained over time. However, no studies have examined in detail which viral genome segments or which regions of segments are maintained. GiPDV-infected transformed cell line IPLB-LdEp/Gi was examined for the persistence of segment F/p157 sequences in order to analyze which sequences were maintained over time in vitro. Restriction endonuclease recognition sites for *Bam*HI, *Xba*I, *Sst*I, *Pst*I, and *Kpn*I were mapped on the segment F/p157 circle shown in Fig. 1A. Corresponding digests of the cloned segment F/p157 circle were shown

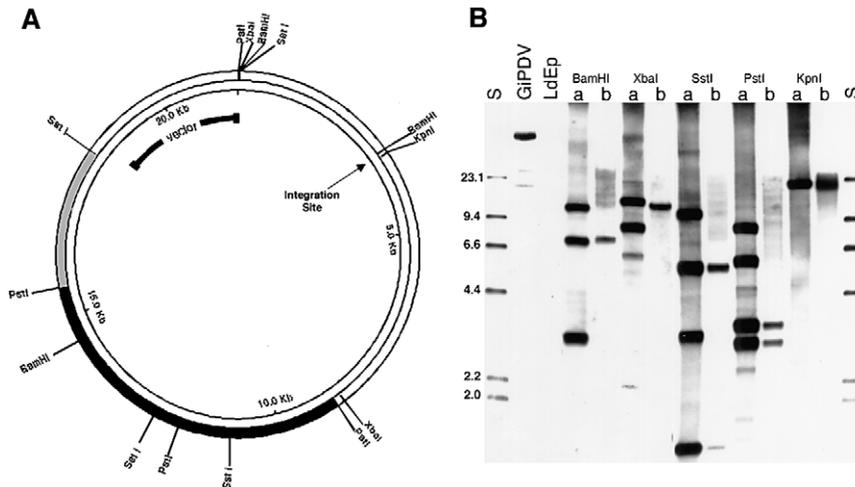


Fig. 1. In vitro maintenance of GiPDV circular ds DNA genome segment F sequences, cloned in its entirety as p157, within infected IPLB-LdEp/Gi cells. (A) Restriction map of GiPDV segment F/p157, generated from full nucleotide sequence of clone p157 (including the plasmid vector sequence) using the MapDraw component of DNASTAR software package. Fragments that were identified within in vitro transformed IPLB-LdEp/Gi chromosomal DNA with all or most restriction endonucleases are shaded black and gray, respectively. The white shaded regions represent *KpnI* and other restriction profiles indicating the presence of the entire segment F/p157 circle within IPLB-LdEp/Gi chromosomal DNA. Segment F/p157 fragment sizes (in kilobase) predicted are: *BamHI*, 11.2, 7.1, & 3.2; *XbaI*, 13.0 & 8.6; *SstI*, 10.9, 5.8, 3.3, & 1.5; *PstI*, 8.8, 6.0, 3.6 & 3.2; *KpnI*, 21.6. (B) Southern blot analysis of transformed GiPDV-infected insect cell line IPLB-LdEp/Gi chromosomal DNA for persisting viral sequences. Uncut genomic DNA from GiPDV and non-infected cells, and DNAs digested separately with restriction endonucleases *BamHI*, *XbaI*, *SstI*, *PstI*, and *KpnI* from clone p157 (GiPDV segment F) (a lanes) and IPLB-LdEp/Gi cell line (b lanes) were electrophoresed through a 0.8% agarose gel and transferred to nylon membrane. Digoxigenin-labeled clone p157 (GiPDV segment F) DNA was used as a hybridization probe. Lane S contains molecular size standard fragments of kilobase sizes as labeled.

in lane **a** for each restriction endonuclease. Digests of IPLB-LdEp/Gi chromosomal DNAs were shown in lane **b**, after hybridization with the labeled cloned segment F/p157 probe. Comparison of IPLB-LdEp/Gi restriction profiles with those of the mapped segment showed that most to all of segment F/p157 was maintained in infected cells. Several IPLB-LdEp/Gi restriction profiles (Fig. 1B, *BamHI*, *SstI*, and *PstI*) indicated the presence of higher molecular weight bands that were not well defined. These were suggestive of fragments that might have been generated from endonuclease digestion at one viral and one chromosomal recognition site, often referred to as off-size fragments. These signs of viral integration were potentially generated at more than one chromosomal locus, but were not seen in association with every restriction endonuclease profile. Restriction fragments present in IPLB-LdEp/Gi were mapped to the circular segment. The regions of the segment F/p157 circle highlighted in black or gray on Fig. 1A identify fragments that were identified within in vitro transformed IPLB-LdEp/Gi chromosomal DNA with the majority of restriction endonucleases. Other restriction profiles indicated the presence of the entire segment F/p157 circle (white shaded on Fig. 1A) within IPLB-LdEp/Gi chromosomal DNA, for example *KpnI* (Fig. 1B) and *EcoRI* (data not shown). These restriction sites occurred in close proximity to the site of viral integration.

The putative site of segment F/p157 integration in infected IPLB-LdEp cells had been identified previously

(Gundersen-Rindal and Dougherty, 2000) and was refined in this study (see Fig. 1B). Most IPLB-LdEp/Gi restriction profiles showed the absence of the segment F/p157 restriction fragment containing the putative site of integration (Fig. 1B). For example, the 11.2 kb *BamHI* fragment containing the putative site of integration is absent, and its viral sequences presumably may contribute to the visible undefined higher molecular weight fragments.

3.3. Sequence analysis of the junction sites of integrated GiPDV DNA

From a previously isolated and sequenced border clone from transformed IPLB-LdEp/Gi cells, the sequence of a single putative site of in vitro integration of segment F/p157 was identified. To analyze events surrounding in vitro integration of GiPDV in Ld cells, additional junction sites were needed. The clone p7-54, which originated from a lambda phage clone containing both viral and host chromosomal sequences and contained the LdEp/Gi putative integration junction described previously, was sequenced to examine the chromosomal sequences in more detail. A second library cloned from transformed IPLB-Ld652Y/Gi cells and screened to isolate junction clones yielded several, among them clone L16XE11. Both *L. dispar* (IPLB-LdEp and IPLB-Ld652Y) junction clones contained GiPDV sequence originating from the GiPDV segment

did not have sequence identity or any identifiable common motif. Studies of PDV integration in the respective wasp host chromosome as a provirus shows that inverted repeated sequences (sometimes lengthy) are present at integration junction sites (Savary et al., 1997). In this example of GiPDV chromosomal integration, palindromic repeated sequences “CATG,GTAC” were identified directly at the site of in vitro integration. Viral sequences of segment F/p157 were characterized by numerous A and T or AT runs.

L. dispar chromosomal sequences in proximity to the site of viral integration in LdEp/Gi clone p7-54 (Fig. 2B) were comparatively unremarkable. Sequence analysis using BLAST (Altschul et al., 1990, 1997) revealed incomplete or limited nucleotide homologies to known genes, for example a 61-nucleotide homology to the vitellogenin gene of *L. dispar*. While clearly chromosomal in origin, this locus was essentially non-coding, particularly in the region upstream of viral integration.

3.4. Structural similarity of Ld652Y viral integration site to retrotransposon

A longer stretch of host chromosomal nucleotide sequence was obtained within IPLB-Ld652Y/Gi junction clone L16XE11 (Fig. 2C) and *L. dispar* sequences in proximity to the site of viral integration in Ld652Y were more informative. Analysis using BLAST of these *L. dispar* chromosomal sequences indicated a small region (underlined) with 3'–5' nucleotide homology to sequences of the *Bombyx mandarina* Pao-like retrotransposon Yamato (GenBank accession AB055223) (Abe et al., 2001), and an open reading frame (ORF) with amino acid homology to the pol protein of *Bombyx mori* (GenBank accession T18196) (Takahashi et al., 1997), encoding a reverse transcriptase and integrase, located just upstream of the chromosomal site of integration (Fig. 2C). These homologies pointed to a potential *L. dispar* retrovirus or retrotransposon. Retroviruses are RNA viruses that are converted to a dsDNA form by reverse transcriptase prior to integration at random sites into the host cell chromosome via an integrase, the protein responsible for integration of viral DNA into host genome(s) (Grandgenett, 1986). Retrotransposons are mobile DNA elements within eukaryote genomes that transpose via RNA intermediates using self-encoded reverse transcriptase and integrase. Retrotransposons have been classified into two groups according to structure, whether they contain long terminal repeats (LTRs) at both ends (LTR type or retrovirus-like element) or not (non-LTR type element) (Eickbush, 1992; Gabriel and Boeke, 1993; Hutchison et al., 1989). Retrotransposons may be integrated at random or at specific sites on the chromosome. No retroviruses or retrotransposons have been identified specifically in association with the gypsy

moth, *L. dispar*, to date, though they are known to exist in nearly every insect and eukaryotic system.

GiPDV segment F/p157, other GiPDV genome segments, or other polydnavirus genome segments sequenced to date do not encode integrase, transposase, polymerase, or other protein(s) that could facilitate self chromosomal insertion in vitro. Furthermore, excision enzymes are unknown for polydnaviruses. The pol gene of retroviruses and retrotransposons encodes a reverse transcriptase followed by an integrase (IN protein) at the 3' end. The pol gene of retroviruses and retrotransposons usually contains a conserved cysteine-histidine motif. In retroviruses, the integrase domain following the reverse transcriptase domain usually contains a HX₃HX_{22–32}CX₂C motif (Johnson et al., 1986) while a CXCX_{8–9}HX₄C integrase motif is generally conserved among divergent species of non-LTR retrotransposons (Jakubczak et al., 1990). In our example of PDV integration in vitro, junction clone L16XE11, *L. dispar* retrotransposon-like chromosomal sequences encoded an ORF for a pol-like protein in which the CXCX_{8–9}HX₄C motif characteristic of non-LTR retrotransposons was present but the spacing between the first C and second C (CXC) was changed to an interval of two bases (CX₂C). This same interval change has been reported for SART1 non-LTR retrotransposons of *Bombyx mori* (Takahashi et al., 1997).

Based on our analysis of GiPDV segment F/p157 integration into two different *L. dispar* cell lines in vitro, a putative integration model is proposed (Fig. 3) in which the palindromic CATG on segment F is recognized and the circular viral segment is integrated via host-encoded reverse transcriptase/integrase.

4. Discussion

Over time, parasitoid wasps have evolved mutualistic relationships with polydnaviruses. The entire virus genome is integrated within the wasp genome as provirus and transmitted vertically, as part of the wasp's genetic material, to progeny. PDVs share some characteristics with transposable elements in that PDV circular genomic segments are integrated within the parasitoid genome and derived by excision from these sites at the onset of viral replication. Prior to excision, integrated proviral segments are characteristically marked by terminal repeated sequences that vary considerably in length, homology, and complexity (Fleming and Summers, 1991; Gruber et al., 1996; Cui and Webb, 1997; reviewed in Webb, 1998; Wyder et al., 2002). The proviral *C. sonorensis* ichnovirus (CSPDV) segments apparently are integrated at multiple loci, flanked on both sides by parasitoid DNA (Fleming and Summers, 1991; Cui and Webb, 1997). In contrast, *Chelonius inanitus* bracovirus (CiV) segments appear clustered and present in

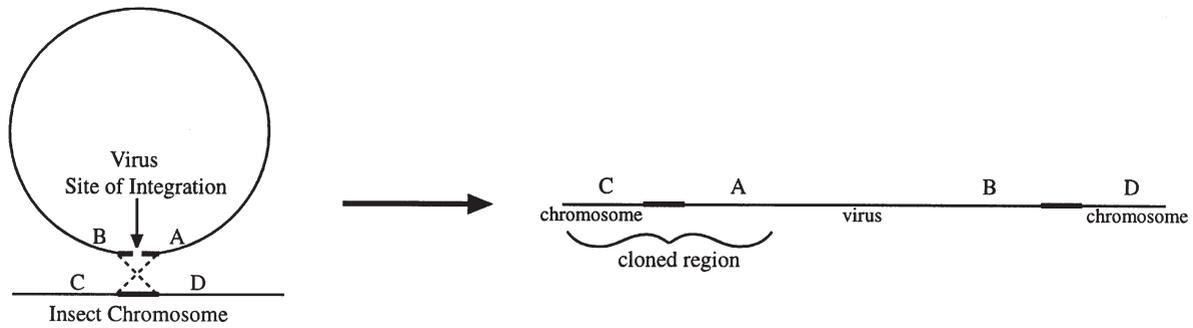


Fig. 3. Putative model for the integration of polydnavirus DNA into insect cell chromosomal DNA as described in the text, suggested by examination of chromosomal:viral junction sites in *Ld* cells. The sequences “CATG” and its palindrome “GTAC” are represented by heavy black lines, with the joined CATGGTAC double length.

tandem array, flanked on both sides by virus DNA (Wyder et al., 2002), as does the EP1 segment of bracovirus *Cotesia congregata* (CcPDV), flanked on one side by parasitoid and the other side by viral segment DNA (Savary et al., 1997). Unlike transposable elements, PDV segment excision occurs only in certain tissues at specific later stages of development, and evidence from at least two PDV systems suggests that segments do not re-integrate into chromosomal DNA (Theilmann and Summers, 1986, 1987; Strand et al., 1992). Excised PDV segments are circularized to form the PDV genomic segments, which, after release from ovarian calyx cells, are injected into parasitized larvae during oviposition to cause host immune-suppression and developmental delays in a manner that favors parasitoid survival. The mutualism between virus and wasp is so complete and ancient that one could consider the provirus segments as legitimate parasitoid genetic material (Whitfield, 2002).

Several laboratories have recently described infection of insect-derived cell lines with PDVs, both IVs and a BV (Kim et al., 1996; McKelvey et al., 1996; Volkoff et al., 1999), and the apparent ability of some PDV sequences to persist in these cells in vitro over time. The phenomenon of PDV in vitro integration has been puzzling because it has been unclear biologically why such an event might occur, and, furthermore, whether the in vitro PDV infections might mirror in vivo processes occurring in PDV-infected larvae. There seem to be numerous potential explanations for PDV sequences integrated in vitro. Among these, PDVs may integrate parts of their genome in a manner that interrupts or affects expression of critical host gene(s). Alternatively, host cells may actively mediate acquisition of PDV sequences with resulting integration into their chromosome(s). While the biological significance of PDV persistence in vitro has not been at all clear, the potential to use PDVs for controlled transformation of insect cells has been appreciated.

For the PDVs examined in vitro to date, the persistence of some, but not all, of the large PDV DNA genome in vitro has been noted. In the GiPDV system, our evi-

dence shows that DNA from at least three different circular genome segments is maintained in vitro. In this study, chromosomal maintenance of a single GiPDV circular segment, segment F, cloned in its entirety as p157 as described previously, was examined in detail. This segment is the best characterized of the GiPDV genome, and was previously shown by this laboratory to be maintained in vitro in transformed IPBL-LdEp cells with evidence strongly supporting chromosomal integration. Here we have shown by mapping experiments that at least a large part of the segment F/p157 circle, in which restriction fragments containing the site of viral integration are “missing” and may contribute to off-size fragments, is consistently maintained chromosomally. The evidence suggests that the entire GiPDV segment F/p157 is maintained in vitro, with restriction profile varying by proximity of the restriction recognition sequence to the site of integration.

Our examination of sequences at the junction site of the same GiPDV circular segment (F/p157) in two different *L. dispar*-derived cell lines has provided the first evidence for a mechanism of PDV integration in vitro. First, F/p157 does not encode integrase, transposase, or any other known protein(s) that can facilitate self insertion (nor do any of the other GiPDV genome segments sequenced to date, Gundersen-Rindal, unpublished) suggesting that a host-derived mechanism is being employed. Also, the insertion on the host genome was different between the two *Ld* lines, but the virus sequence junctions were the same in each. This suggests the actual host insertion site may be random but that a specific GiPDV sequence is being recognized. A clue for the mechanism also comes from our examination of junction clone L16XE11, in which Ld652Y chromosomal sequences adjacent to the site of integration were characteristic of a *L. dispar*-associated retrovirus or retrotransposon. These sequences encoded an ORF for a pol-like protein in which an integrase domain, CXCX₈ϕHX₄C, conserved among non-LTR retrotransposons, as well as some group II introns, was identified. The pol gene of retroviruses and retrotransposons encodes two

domains: a reverse transcriptase followed by an integrase. Reverse transcriptase is able to act on DNA template and initiate a double strand break. The integrase protein uses linear DNA as its template to form a pre-integration complex and on a 5'CA(OH) 3', on a phosphodiester bond of DNA target, initiates a strand transfer reaction. One can envision a mechanism (Fig. 3) where specific target site sequences on the GiPDV circular segment F/p157, namely "CATG", are recognized by reverse transcriptase/integrase and a double strand break initiated. This would be followed by the *L. dispar* retrotransposon-like element integrase acting "in trans" to mediate ds DNA joining to result in the linearized and integrated GiPDV segment, with the 8-base pair "CATGGATC" site duplicated on each end.

Furthermore, the evidence supports the integration of the full segment F/p157 in vitro in both Ld cell lines. We suggest the "CATGGTAC" immediately at the site of integration may represent a 4-base pair palindromic direct repeat sequence for this segment and a recognition site for both recombination and in vitro integration. This sequence could potentially represent the circularization point for the segment after its excision from the parasitoid genome. Palindromic termini to LTR sequences have been identified in other PDV systems, in most detail by Cui and Webb (1997) in which the segment W of CsPDV identical 1.2 kb LTRs present at the site of proviral integration were shown to terminate in palindromic sequences of 4-base pair length, "GATC". These palindromic LTR termini sequences were then suggested to be sites for recombination of the segment W to form nested segments R and M. The direct repeats present on proviral termini of bracoviruses identified in CiV and CcPDV have been much shorter, from 14 to 24 base pairs (Savary et al., 1997; Wyder et al., 2002), as we would similarly expect for GiPDV. We may predict the sequences "CATG" and its palindrome "GTAC" may represent short repeated sequences from the site of GiPDV segment integration in the *G. indiensis* parasitoid. The excision of the segment F from the parasitoid genome at these sites, followed by circularization to form the circular segment F, could result in their joining to form the "CATGGTAC" seen in the circular segment F. Thus the "CATGGTAC" palindromic repeat present in viral sequences directly at the site of in vitro integration would represent a recognition site for viral integration by virtue of structure and sequence. In vitro linearization and integration of segment F would directly mirror the integrated proviral state of the integration in the parasitoid in vivo. However, we must examine GiPDV segment F integrated in the parasitoid host as provirus to test this prediction.

From an evolutionary standpoint, a virus such as PDV with an integrated provirus-excision-circularization life-style might be expected to also integrate segments in host cells and that these could be maintained in similar

fashion as a provirus. Furthermore, PDVs in general self-encode very few proteins related to their replication, but, rather, seem to rely on host machinery (Webb, 1998). Thus, such a mechanism where virus is integrated using host-encoded gene products (presumably, in this system, *L. dispar* retrotransposon pol gene-encoded reverse transcriptase/integrase acting "in trans") would fit well within the unusual life cycle evolved by polydnviruses. In addition, such a mechanism offers important clues into how polydnviruses might disassemble and propagate themselves during the cycle of infection.

The ability of all PDVs as a general phenomenon, both IVs and BVs, to infect and essentially permanently transform insect cell populations in vitro has yet to be investigated. If the phenomenon of integration of PDV sequences in vitro is related evolutionarily to the nature of the virus it seems logical that some or all PDVs would exhibit a similar ability to persist in vitro. This remains to be seen, as not all cell lines infected with PDVs in vitro and examined for evidence of viral persistence have shown evidence of chromosomal maintenance. GiPDV remains the first and, to date, only BV shown capable of in vitro integration. Since IVs and BVs do not share a common evolutionary origin (Whitfield, 1997, 2002), suggesting they were derived independently from different ancestral viruses, in vitro viral maintenance and other features sometimes exhibited in common by both genera of PDVs may be similar manifestations resulting from common viral life cycles, or similarly evolved viral recognition mechanisms.

We have described a putative in vitro integration mechanism for one segment of the GiPDV DNA genome. It is necessary to examine the other GiPDV genome segments for which we have evidence of maintenance in transformed insect cells to further develop a model for in vitro integration and propagation of PDVs. In addition, we would like to examine GiPDV in vitro integration in numerous other infected insect cell lines derived from a variety of insect hosts. Stable integration of exogenous GiPDV sequences into the insect cell nuclear genome in vitro is highly significant for its potential to be used for continuous transformation of insect cells. For the future, it will be essential to investigate whether or not PDV sequences may be similarly integrated in insect cells in vivo.

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